Fish and Shellfish Immunology 86 (2019) 713–723

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Oestrogen differentially modulates lymphoid and myeloid cells of the European sea bass *in vitro* by specifically regulating their redox biology

Matthieu Paiola, Catarina Moreira, Aurélie Duflot, Thomas Knigge, Tiphaine Monsinjon*

Normandy University, FR CNRS 3730 SCALE, UMR-I 02 INERIS-URCA-ULH Environmental Stress and Aquatic Biomonitoring (SEBIO), Université Le Havre Normandie, F-76600, Le Havre, France

ARTICLE INFO

Keywords:
Leucocytes
Oestrogen receptor
Oestradiol
Thymus
Spleen
Head-kidney
Reactive oxygen species
Reactive nitrogen species

ABSTRACT

Besides their obvious role in sex determination and reproduction, oestrogens display a prominent and complex immunomodulatory role across all vertebrates. To date, our knowledge on the oestrogenic immunomodulation in non-mammalian species is, however, scarce. In both teleosts and mammals, the direct immunomodulatory function of oestrogen is underscored by the presence of multiple oestrogen receptor subtypes in the various immune cells. For a better understanding of the regulatory processes, we investigated the oestrogen receptor expression in two major lymphoid organs of European sea bass: the head-kidney and the spleen. All oestrogen receptor subtypes, including nuclear and membrane oestrogen receptors, were present in both immune organs as well as in the isolated leucocytes. The same findings have been previously made for the thymus. To determine the oestrogen responsiveness of the different immune cell populations and to evaluate the importance of non-genomic and genomic pathways, we assessed the kinetics and the concentration dependent effects of 17β-oestradiol on isolated leucocytes from the head-kidney, the spleen and the thymus *in vitro*. Given the importance of reactive oxygen species as signalling and defence components in mammalian immune cells, the oxidative burst capacity, the redox status and the viability of both lymphoid and myeloid cells were measured by flow cytometry. The treatment with 17β-oestradiol specifically modulated these parameters depending on (1) the time kinetic, (2) the concentration of 17β-oestradiol, (3) the immune cell population (lymphoid and myeloid cells) as well as (4) the lymphoid organs from which they originated. The observed *in vitro* oestrogenic effects as well the presence of various oestrogen receptor subtypes in the immune cells of sea bass suggest a complex and direct oestrogenic action *via* multiple interconnected oestrogen-signalling pathways. Additionally, our study suggests that the oestrogenic regulation of the sea bass immune function involves a direct and tissue specific modulation of the immune cell redox biology comprising redox signalling, NADPH-oxidase activity and H_2O_2-permeability, thus changing oxidative burst capacity and immature T cell fate because oestrogen impacted thymocyte viability. Importantly, immune cells from both primary and secondary lymphoid organs have shown specific *in vitro* oestrogen-responsiveness. As established in mammals, oestrogen is likely to be specifically and directly involved in immature T cell differentiation and mature immunocompetent cell function in sea bass too.

1. Introduction

To eliminate pathogenic agents and abnormal cells, vertebrates have developed in addition to the innate immune system a second line of defence: the adaptive immune system, also referred to as the specific immune system [1,2]. Generally, innate and adaptive components are associated to the myeloid and lymphoid cell lineages, respectively. Distinct progenitors and antigen binding receptors determine their function: innate immune cells express a germline-encoded antigen-binding receptor, the so-called pathogen recognition receptors, whereas lymphocyte antigen recognition is based on clonally expressed antigen receptor genes, the elements of which are assembled in various combinations [1,2]. Both types of immune cells are essential for the immune system, as they have complementary and non-redundant functions.

Abbreviations: E2, 17β-oestradiol; FSC, forward scatter; H_2DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; H_2O_2, hydrogen peroxide; MFI, mean fluorescent intensity; $NO^{\bullet}$, nitric oxide; $O_2^{\bullet-}$, superoxide anion; $OH^{\bullet}$, hydroxyl radical; PMA, phorbol 12-myristate 13-acetate; ROS, reactive oxygen species; RNS, reactive nitrogen species; SSC, side scatter; Treg, regulatory T cell

* Corresponding author. UMR-I 02 INERIS-URCA-ULH, Unité Stress Environnementaux et Biosurveillance des milieux aquatiques, UFR Sciences et Techniques, Université Le Havre Normandie, 25 rue Philippe Lebon, 76063, Le Havre, France.

E-mail address: tiphaine.monsinjon@univ-lehavre.fr (T. Monsinjon).

https://doi.org/10.1016/j.fsi.2018.11.078
Received 13 September 2018; Received in revised form 23 November 2018; Accepted 30 November 2018
Available online 01 December 2018

Indeed, innate immune cells constitute sentinel cells: during an invasion of pathogenic agents, they rapidly trigger the immune response and eliminate the pathogenic agent. As a second line of defence, innate immune cells, and especially antigen-presenting cells, activate lymphocytes in secondary lymphoid organs to induce a specific immune response [3–6].

In many aspects, the innate and adaptive immune system of teleosts bears resemblance to that of mammals. The adaptive component is based on two lineages of lymphocytes with distinct functions, the T and B cells. Basically, T cells coordinate the immune response and eliminate transformed cells. B cells, on the other hand, are involved in the humoral defence by the secretion of specific and natural (*i.e.*, unspecific) antibodies [1,7,8]. Immune cells of the innate component, such as macrophages, mast cells, granulocytes, and dendritic cells are being retrieved in teleost fish [3,4,9,10]. Moreover, major immune organs appear to be evolutionarily conserved. The thymus, which is the primary site of T cell development, and the spleen, a major secondary lymphoid organ, are present in all vertebrate classes and may, therefore, be considered evolutionary ancient immune organs [1,11]. Differences exist, however, in the site of myelopoiesis and B cell development. In mammals this takes place in the bone marrow, whereas in teleosts these cells develop in the head-kidney [1,11].

In both, teleosts and mammals, the immune system is tightly regulated by endocrine signals in conjunction with the reproductive system [12–15]. This crosstalk has been relatively well characterised in mammals with respect to the modulation of immune performance and autoimmune disease severity during pregnancy and menstrual cycles [16–18]. Notably, the female sex hormone oestrogen has been investigated for its immunoregulatory function. Indeed, this steroid hormone is well known to modulate innate and adaptive immune cell function across all vertebrate classes [12–15]. Given the considerable degree of evolutionary conservation between the immune system of higher and lower vertebrates as well as the potential key role of oestrogen in the reproductive-immune trade-off in vertebrates, one may even assume the immunomodulary role of oestrogens in fish to have a similar evolutionary origin than those in mammals [13,19,20]. The cellular action of oestrogen is mediated through both, genomic and non-genomic signalling pathways. These pathways either imply the nuclear oestrogen receptors (Esrs), which belong to the superfamily of the ligand-dependent transcription factors, or a membrane-associated oestrogen receptor comprising membrane Esrs and G-protein-coupled oestrogen receptors (Gpers). A broad and varied expression of these oestrogen receptors can be found in the immune organs and immune cells of fish, a situation similar to that observed in mammals [12,15,21]. The occurrence of both membrane and nuclear oestrogen receptor isoforms in the immune system suggests that oestrogens are likely to have complex immunomodulatory roles, both in mammals and in teleosts. In fact, in mammals, oestrogens have a context-dependent action on immune cell function, *i.e.* their action depends on multiple parameters, such as tissue and cell type as well as the oestrogen concentrations and the oestrogen receptor subtypes expressed [14,17,22].

Among the innate component, the professional vertebrate phagocytes (granulocytes and macrophages) use the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) as major means to kill invading microorganisms [3,4]. In phagocytes, for instance, membrane-bound NADPH-oxidase and inducible nitric oxide ($NO^{\bullet}$) synthase generate large amounts of superoxide anion ($O_2^{\bullet -}$) and $NO^{\bullet}$, respectively. This process is commonly referred as oxidative or respiratory burst [9,23,24]. In macrophages, mitochondrial-derived ROS also participate in the microbicidal activity [24]. Importantly, the ROS, but also the $NO^{\bullet}$, serve as second messengers by activating transcription factors, protein tyrosine kinases or phosphatases. They are, therefore, essential to the intracellular signalling pathways [25–28]. In the mammalian immune system, ROS- and NO-signalling are particularly important for the differentiation, activation and/or apoptosis of numerous immune cell types such as T and B cells, but also of natural killer cells, dendritic cells, and macrophages [23–25,27].

In the European sea bass, *Dicentrarchus labrax,* we recently observed *in vivo* that 17β-oestradiol (E2) inhibited oxidative burst capacity in the spleen [19]. Based on the expression of various genes implicated in T cell maturation, the inhibition of oxidative burst was suggested to result from a stimulation of regulatory T cell (Treg) differentiation and activity, similar to the situation observed in mammals [19,22]. By contrast, E2 neither had an effect on the oxidative burst capacity, nor on Treg differentiation in the head-kidney. To test whether the results obtained *in vivo* did not derive (1) from a general inability of E2 to directly modulate head-kidney leucocytes due to the lack of receptor mediated signalling or (2) from the incapacity of E2 to modulate the oxidative burst capacity of the head-kidney leucocytes, we investigated the oestrogen receptor expression in spleen and head-kidney as well as in their isolated leucocytes. Similar to earlier observations in the thymus [20], both, membrane and nuclear oestrogen receptors were found to be expressed in head-kidney as well as in spleen leucocytes. Based upon this information, we assessed the kinetics and concentration effects of E2 on the oxidative burst capacity *in vitro*, as well as the redox level and the viability of leucocytes from the head-kidney, the spleen and the thymus.

2. Material and methods

2.1. Animals and sampling

Sea bass (*Dicentrarchus labrax*) fingerlings from the hatchery at Gravelines, France, were raised at Aquacaux, Octeville, France, in 1800 L tanks with a continuous flow of filtered and aerated marine seawater at environmental temperatures. The fish were fed daily *ad libidum* with “Turbot label rouge” fish feed (Le Gouessant, Lamballe, France). All fish were handled in accordance with the European Union regulations concerning the protection of experimental animals (Dir 2010/63/EU).

Two batches of fish were employed for this study as characterised in the following: five fish of one-and-a-half-year of age with a total length of 21.8 cm ± 2.9 standard deviation (s.d.) and a weight of 97.8 g ± 10 s.d. (group 1) for molecular biology as well as 15 fish of two-years of age for the isolation of leucocytes from head-kidney, spleen and thymus used for *in vitro* exposures. These fish had a total length of 25.8 cm ± 2.9 s.d. and a weight of 252 g ± 36 s.d. (group 2). Although both populations generally consisted of sexually immature males and females, the gender of some of the older fish (group 2) could be determined by macroscopic observation, resulting in a sex ratio of 1.5 (F/M). Specimens of group 1 were sacrificed in June 2015 and specimens of group 2 in November 2016.

The fish were anesthetized with tricaine methanesulfonate (MS 222; Sigma, St. Louis, USA) in order to measure weight and total length before sacrificing them by an overdose of MS 222. Following decapitation, thymus, head-kidney and spleen were dissected. For PCR analysis of esr- and gper-isoforms (group 1), one part of the head-kidney and spleen were directly snap frozen in liquid nitrogen, whereas leucocytes were isolated from the other part and resuspended in lysis buffer (RLT buffer, RNeasy kit, Qiagen, Hilden, Germany). Both, leucocytes and whole tissue, were equally stored at −80 °C for subsequent RNA-extraction. For the primary cell cultures (group 2), entire thymuses, spleen and head-kidneys were used for leucocyte isolation.

2.2. Leucocyte isolation

All solutions for leucocyte preparation were adjusted to 360 mOsm/kg. Dissected thymuses, head-kidney and spleen were immersed in cold Leibovitz medium (L15; Sigma) and forced through a 100 μm cell strainer. The cell suspension was centrifuged at 1200 *g* for 5 min at 4 °C. The supernatant was discarded and the cells were resuspended in L15 and filtered through a 40 μm mesh before loading on a Ficoll gradient

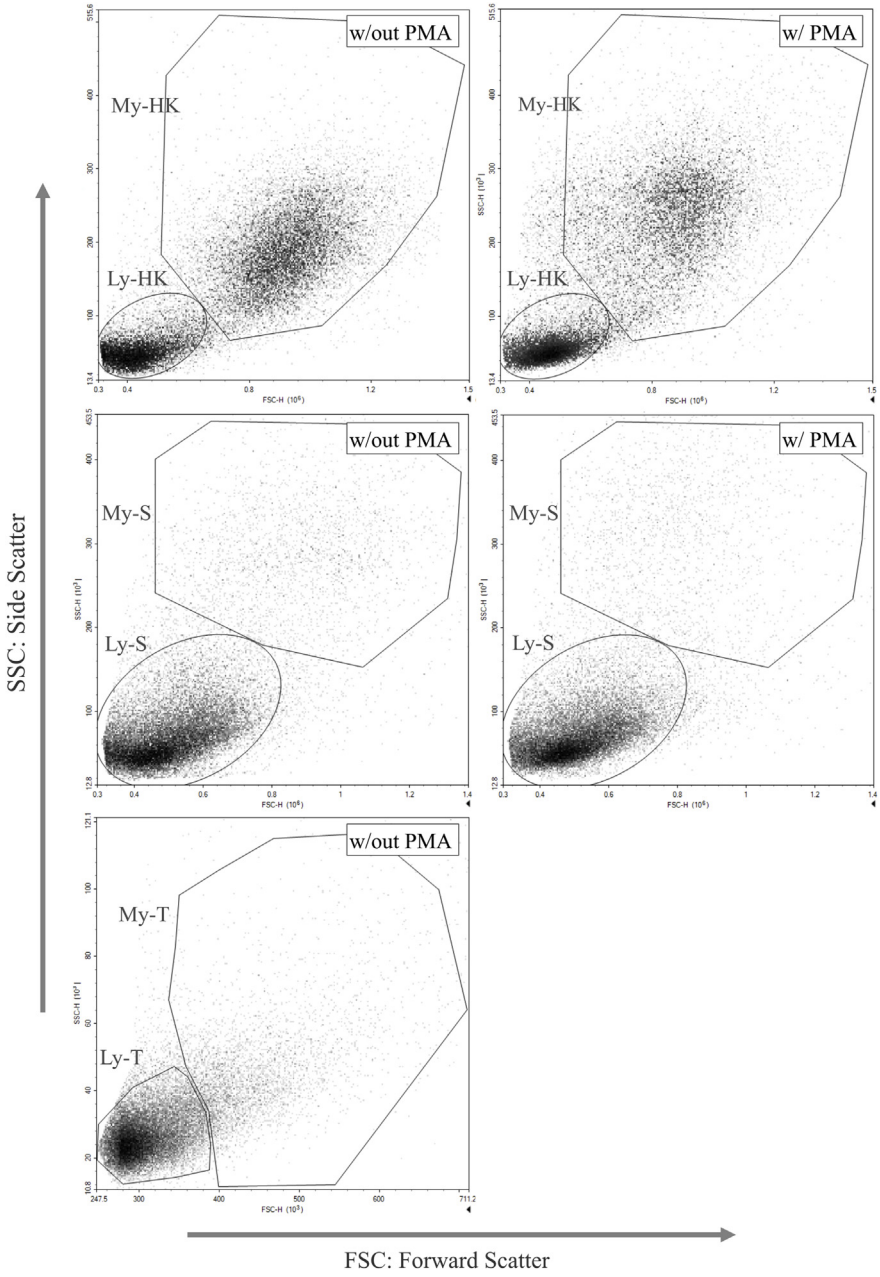

Fig. 1. Representative Side scatter (SSC)/Forward scatter (FSC) flow cytometric profiles of leucocytes isolated from the head-kidney (HK), spleen (S) and thymus (T). Lymphoid (Ly) and myeloid (My) cells were analysed separately by gating cell populations according to their size and granularity. A, C and E show profiles of isolated leucocytes from head-kidney, spleen and thymus in basal condition; B, D show profiles of leucocytes isolated from the head-kidney and spleen in PMA-stimulated condition.

(Pancoll, PAN™ BIOTECH, Germany) at a density of 1.077 g/mL followed by a centrifugation at 400 *g* for 30 min at 4 °C. The leucocytes were collected at the interface between the L15 and the Ficoll, washed and centrifuged twice with L15 at 1200 *g* for 5 min and 4 °C. For PCR-analyses, erythrocytes were lysed before loading the leucocytes on the Ficoll gradient in order to ensure cell purity. To this end, cellular solutions were incubated with ammonium chloride-Tris solution for 30 min at room temperature under constant stirring. Subsequently, leucocytes were concentrated by centrifugation at 1200 *g* for 8 min and washed once with L15 centrifugation at 1200 *g* for 5 min at 4 °C. For flow cytometry, this step was not necessary and, therefore, erythrocyte lysis was omitted for all organs in the exposure experiments. The cells were stored at 4 °C in L15 overnight before cell exposure.

2.3. RNA-extraction and polymerase chain reaction

Four biological replicates were performed for each RT-PCR. Extraction of RNA was conducted using standard procedures as described previously [20]. Briefly, total RNA was obtained from whole tissue utilizing the Tri Reagent (Sigma) by homogenisation in Precellys® tubes (CK14; Bertin instruments, Montigny-le-Bretonneux, France) twice for 10 s at 5000 rpm and subsequent centrifugation at 12,000 *g* for 15 min at 4 °C to eliminate debris. RNA-extraction from isolated leucocytes was performed with the RNeasy kit (Qiagen) according to the supplier's instructions. After RNA-extraction, possible DNA-contamination was removed by digestion with the TURBO DNA-*free* Kit (Invitrogen-Ambion, Carlsbad, USA) according to the supplier's recommendation. RNA-quality was assessed on 1% (w/v) agarose gels and the yield was quantified with a Nanodrop One (Thermo Fisher

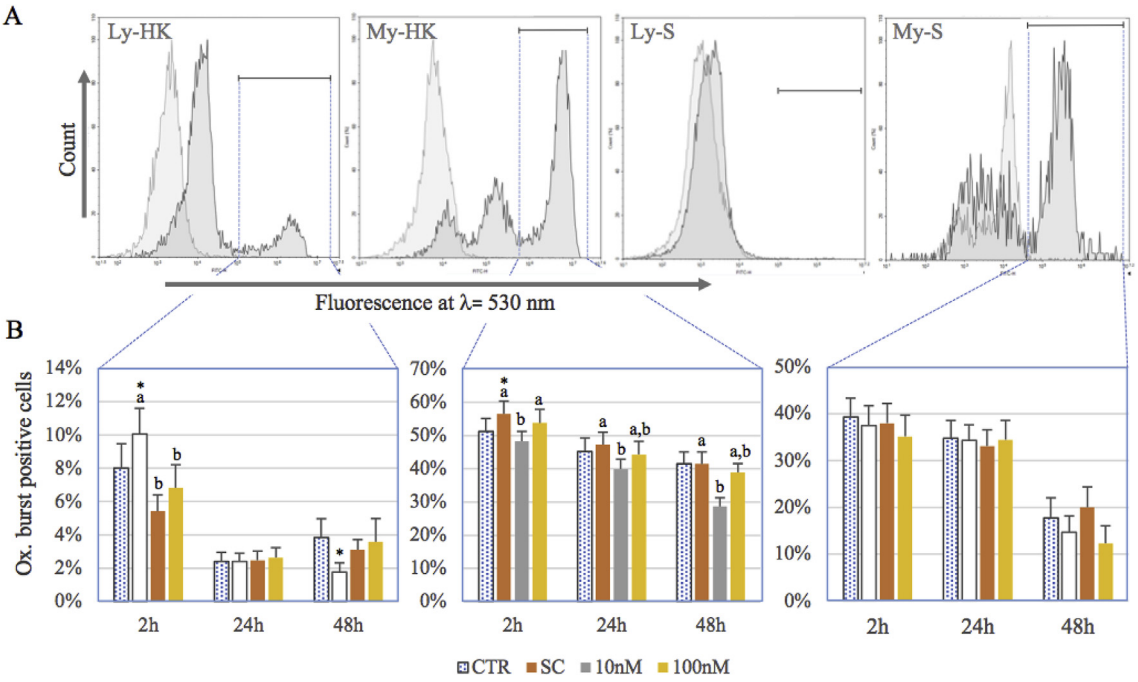

Fig. 2. Flow cytometric gating strategy to evaluate the effect of 17β-oestradiol on oxidative burst capacity: A, histogram in the FL1-channel (λ = 530 nm) of unstimulated (pale grey) and stimulated (dark grey) lymphoid and myeloid cells from the head-kidney (HK) and spleen (S). Gates selecting the "oxidative burst positive cells", *i.e.*, the peaks with the highest fluorescence are situated on the right-hand side. B, proportion of oxidative burst positive lymphoid (Ly) and myeloid (My) cells after 2 h, 24 h and 48 h of primary cell culture without treatment (CTR), with 0.00005% ethanol (*i.e.*, solvent control, SC), with 10 nM and with 100 nM 17β-oestradiol. Values are means ± standard error of the means; *, significantly different between the CTR and SC cells at $p < 0.05$; different letters indicate statistically significant differences between SC, 10 nM and 100 nM groups at $p < 0.05$.

Scientific, Waltham, USA). Samples were stored at −80 °C until further processing.

Reverse transcription and PCR were performed as in Paiola et al. (2017). The M-MLV Reverse Transcriptase H$^-$ (Promega, Madison, USA) was incubated with 1 µg of total RNA and an oligo(dT)15 primer for 10 min at 40 °C, followed by 60 min at 45 °C and 15 min at 70 °C. The cDNA was stored at −20 °C until performing PCR with oestrogen receptor subtype specific primers. For *esr1*, *esr2a* and *gpera*, Purple Taq (Ozyme, Montigny-le-Bretonneux, France) and for *esr2b* and *gperb*, Platinum® Taq DNA Polymerase (Thermo Fisher Scientific) was used according to Paiola et al. (2017). Negative controls were performed with DNA free water. The size of the different amplicons was verified on 2% agarose gels.

2.4. Leucocyte exposure

Whole isolated leucocytes were adjusted to 2.5 × 10^6 cells/mL and cultured in 96 well plates with 250,000 cells/well in modified L15 with 10% of heat-inactivated foetal bovine serum (Sigma) and 1% of penicillin/streptomycin (Gibco, Thermo Fisher Scientific). Depending on the condition, isolated leucocytes were cultured in modified L15 at 16 °C without the addition of solvent and E2 (CTR), with solvent alone (0.00005%, v/v of ethanol) as a solvent control (SC) as well as with nominal concentrations of 10 nM and 100 nM of E2 (E8875; Sigma) in 0.00005% (v/v) ethanol for 2 h, 24 h and 48 h. The ethanol was reduced to a minimum, as solvents may modulate immune cell parameters like oxidative burst capacity [29].

2.5. Flow cytometry

For the measurement of physiological parameters, such as viability, cell population distribution and ROS-production, the cellular concentration was adjusted to 1 × 10^6 cells/mL at the end of the respective culture/exposure periods. To this end, modified L15 supplemented with the corresponding compound was added and the cells were mixed by gentle pipetting. From whole leucocyte fraction the flow cytometric analysis allows the distinction of two leucocyte subpopulations: the myeloid and lymphoid cells based on their granularity, *i.e.*, their internal complexity corresponding to the side scatter (SSC) and their size corresponding to the forward scatter (FSC). According to previous work [30,31], a population of myeloid cells with larger size (*i.e.*, FSC) and granularity (*i.e.*, SSC), as well as a population of lymphoid cells being smaller and less complex were identified (Fig. 1), which allows, after gating, the analysis of physiological parameters on both myeloid and lymphoid cells. Each flow cytometric measurement was conducted with 25,000 events in the gate "cells" comprising both cell types. The population distribution for each cell type corresponded to the percentage of events in either of the two gates ("lymphoid cells" and "myeloid cells"). Lymphoid and myeloid cell mortality was estimated with 50 µg/mL propidium iodide (Sigma) in obscurity over 10 min at room temperature. The mortality of each subpopulation represents the proportion of lymphoid or myeloid cells with high red fluorescence at a wavelength between λ = 615 and 620 nm. All samples with a mortality exceeding 10% were excluded from further analyses. The ROS-levels and respiratory burst were assessed using 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA, ThermoFisher) and phorbol 12-myristate 13-acetate (PMA, Sigma) as described by Bado-Nilles et al. (2014) and Haugland et al. (2014) [33]. H_2DCFDA is an unspecific oxidative-sensitive fluorescent dye, which reacts with both RNS and ROS. It is commonly used to measure intracellular ROS and oxidative burst capacity in flow cytometry [34–36]. For stimulation, PMA, which is an analogue of diacylglycerol and thus an activator of the protein kinase C, was used to activate the NADPH-oxidase and to provoke oxidative burst in phagocytes [9,32,37]. The cells were incubated with 5 µM of H_2DCFDA for 30 min followed by a stimulation using 2 µg/mL of PMA for 30 min. The basal level of ROS and RNS combined was established for each sample by omitting the PMA-stimulation and related to the fluorescence measured with PMA that derives from ROS only. The

capacity of oxidative burst was measured for each gate (lymphoid and myeloid cells) as performed by Peluso et al. (2012) [38] and Rabe-sandratana et al. (1992) [39]. Four distinct parameters were analysed: the mean fluorescence intensity (MFI) in the basal condition, the MFI under stimulated condition, the ROS-level index and the proportion of oxidative burst positive cells. The ROS-level index is based on the population MFI (arbitrary units) and was previously used to evaluate the oxidative burst capacity [19,32]. The ROS-level index was calculated by dividing the stimulated cell MFI by the unstimulated cell MFI. To evaluate the oxidative burst capacity, the proportion of oxidative burst positive cells was estimated using gates that select "oxidative burst positive cells" with a high fluorescence intensity at $\lambda = 530$ nm (Fig. 2), as previously performed by Haugland et al. (2014) [33]. To quantify the "oxidative burst positive" cells only, the proportion of gated myeloid/lymphoid cells in the unstimulated condition was subtracted from the proportion of "oxidative burst positives" myeloid/lymphoid cells in the stimulated condition. All flow cytometric measurements and their corresponding data analyses were carried out with NovoCytes™ (ACEA Biosciences Inc., San Diego, USA) and NovoExpress® software (ACEA Biosciences Inc.).

2.6. Statistical analysis

Statistical analyses were conducted with SigmaPlot (version 12.0, Systat Software Inc., San Jose, USA). The results of flow cytometry are reported as histograms with means and standard errors (s.e.). Prior to analyses, outliers were eliminated using the Grubb's outlier test (for combined data from CTR- and E2-group, ©2017 GraphPad Software Inc., La Jolla, USA). The datasets were checked for normality and equal variances using the Shapiro-Wilk test and the Median-Levene's test, respectively. For comparing gender differences, if normal distribution and homoscedasticity could be confirmed, one-way analysis of variance (ANOVA) was used for parametric hypothesis testing; otherwise non-parametric one-way ANOVA on ranks (Kruskall-Wallis) was conducted. The immune physiological parameters did not show a significant sex-dependent effect, thus data from males and females where combined. Therefore, to compare culture conditions at each incubation time, one-way repeated measures ANOVA or, alternatively, non-parametric one-way repeated measurements ANOVA on ranks (Friedman) was conducted. A repeated measurements ANOVA was performed for each related individual to detect differences between conditions for each incubation time. The ANOVA-test was followed by a Tukey's *post-hoc* test and the Kruskall-Wallis test was followed by Dunn's test. The results were considered significantly different at an α-level of 5% ($p < 0.05$).

3. Results

3.1. Polymerase chain reaction

Both, head-kidney and spleen expressed all transcripts of the nuclear receptors *esr1, esr2a, esr2b* (Fig. 3). As for the membrane receptors, *gpera* produced a weak band in the spleen, whereas no expression was detected in the head-kidney. Contrariwise, the *gperb* displayed a well detectable level of expression in both organs (Fig. 3).

In the isolated leucocytes from the head-kidney, the transcripts of *esr1* and *gperb* were well detected. For *esr2a* and *esr2b,* a weak band was visible in all samples. No amplification for *gpera* could be observed for the majority of the fish. The spleen leucocytes produced well-defined *esr1*-and *esr2a*-amplicons. By contrast, *esr2b*-amplicons were well represented in half of the fish only, but barely detectable in the other specimens and these interindividual differences were not correlated with the sex of the fish. Furthermore, *gperb*-amplification resulted in a weak, but well detectable band, whereas *gpera*-transcripts produced merely very weak bands.

3.2. Effect of E2 on immune cell parameters

Despite the reduced amount of solvent, we observed minor, yet statistically significant effects on the measured immune parameters when comparing non-treated cells to the solvent controls. Indeed, ethanol increased the respiratory burst capacity and ROS-level index of leucocytes isolated from the head-kidney (Figs. 2 and 4). It also slightly modulated the leucocyte mortality (Fig. 6) and decreased the proportion of macrophages (Fig. 7). Therefore, effects of E2 on the various immune cell parameters were assessed by comparing the E2-exposure to the solvent control only.

3.2.1. Redox status and oxidative burst capacity

3.2.1.1. Two hours of E2-exposure. In the head-kidney, the E2-treatment at 10 nM significantly decreased the proportion of oxidative burst positive cells (Fig. 2) from 10.1% ± 1.55 s.e. to 5.42% ± 0.98 s.e. for lymphoid cells ($p < 0.001$) and from 56.5% ± 3.86 s.e. to 48.2% ± 3.05 s.e. for myeloid cells ($p < 0.001$). At 100 nM, E2-treatment also decreased the proportion of oxidative burst positive cells from 10.1% ± 1.55 s.e. to 6.84% ± 1.38 s.e. in lymphoid cells ($p < 0.001$) and 56.5% ± 3.86 s.e. to 53.7% ± 4.11 s.e. for myeloid cells. The latter was, however, not statistically significant ($p = 0.161$). Considering the ROS-level indices (Fig. 4), they were significantly decreased by the E2-treatment at 10 nM from 62.2 ± 9.7 s.e. to 28.9 ± 3.5 s.e. ($p < 0.001$) for lymphoid cells, and from 430.0 ± 60.6 s.e. to 331.0 ± 39.8 s.e. ($p = 0.046$) for myeloid cells, as shown in Fig. 4. At 100 nM the treatment equally decreased the ROS-level index of the lymphocytes from 62.2 ± 9.7 s.e. to 47.4 ± 7.6 s.e. ($p = 0.025$), but had no effect on myeloid cells of the head-kidney

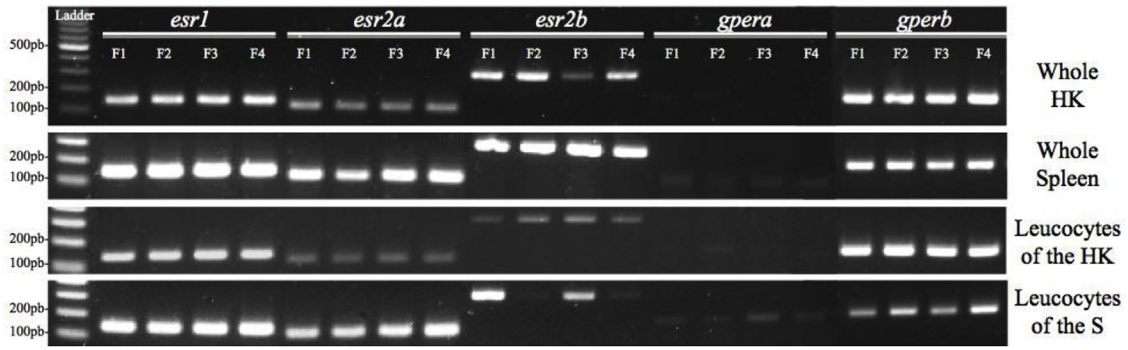

Fig. 3. PCR-amplicons of the oestrogen receptors *esr1, esr2a, esr2b, gpera* and *gperb* in whole tissue extracts of head-kidney (HK) and spleen (S) as well as in isolated leucocytes from the respective organs.

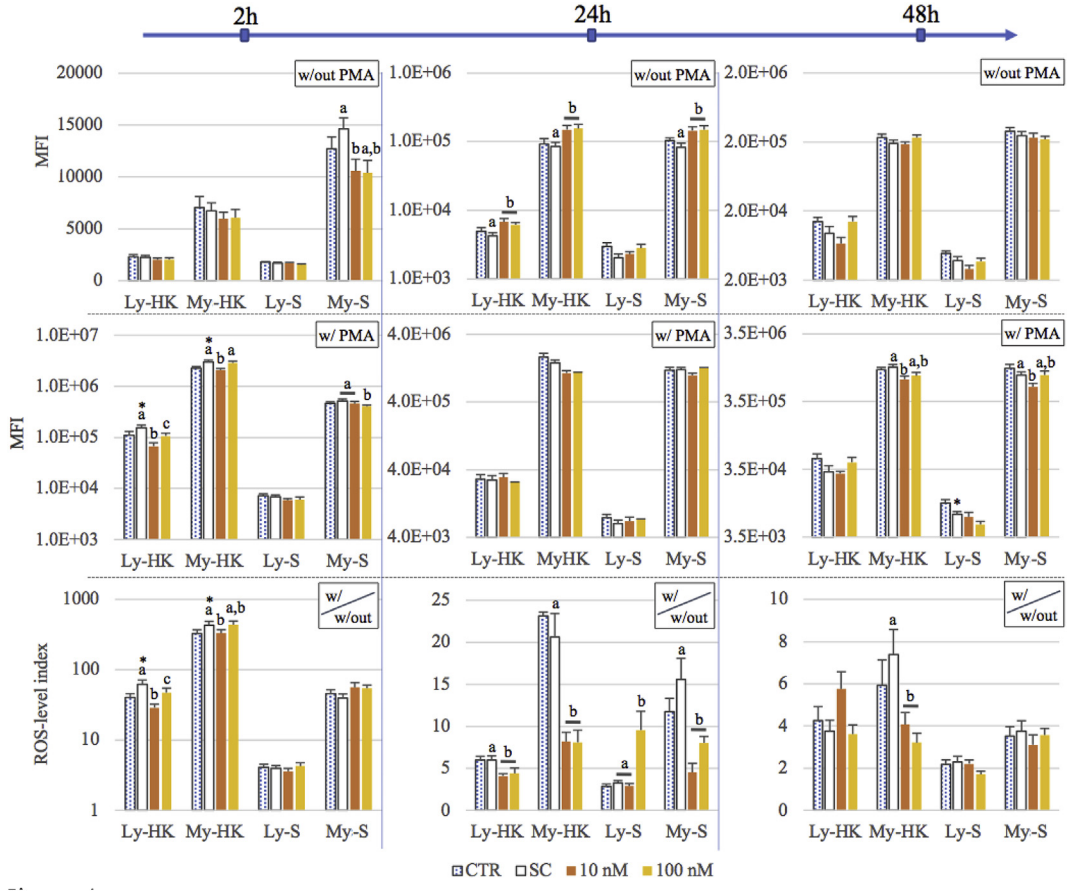

Fig. 4. Basal ROS-level (w/out, without PMA) and PMA-stimulated ROS-level (w/, with PMA) and ROS-level index (basal ROS-level divided by the stimulated ROS-level) of leucocytes isolated from the head-kidney (HK), the spleen (S) measured in lymphoid (Ly) and myeloid (My) cells after 2 h, 24 h and 48 h of primary cell culture without treatment (CTR), with 0.00005% ethanol (*i.e.*, solvent control, SC), with 10 nM and 100 nM 17β-oestradiol. MFI, mean fluorescence index (for details see text). Values are means ± standard error of the means; *, significantly different between the CTR and SC cells at $p < 0.05$; different letters indicate significant differences between SC, 10 nM and 100 nM groups at $p < 0.05$.

($p = 0.997$). The diminished ROS-level index is likely to be due to a significant, E2-mediated decrease of PMA-stimulated ROS-levels (Fig. 4) at 10 nM (from 153,299 ± 20,462 s.e. to 67,327 ± 10,993 s.e., $p < 0.001$ for the lymphoid cells and from 2,974,950 ± 287,733 s.e. to 2,074,639 ± 164,368 s.e. $p < 0.05$ for the myeloid cells) and at 100 nM (from 153,299 ± 20,462 s.e. to 106,737 ± 14,567 s.e., $p < 0.001$ for the lymphoid cells only). E2 did, however, not significantly modulate the basal ROS/RNS-level in both leucocyte populations of the head-kidney (Fig. 4).

In the spleen, E2 did not significantly affect the proportion of oxidative burst positive cells or the ROS-level indices (Figs. 2 and 4). However, 10 nM E2, but not 100 nM, significantly decreased the basal ROS/RNS-level of the myeloid cells (from 14,639 ± 1,053 s.e. to 10,561 ± 1,146 s.e., $p = 0.009$); no such effect was observed in lymphoid cells (Fig. 4). Considering the stimulated ROS-level (Fig. 4), E2 at 100 nM had a significant effect on the myeloid cells only, in which it decreased the MFI from 511,711 ± 55,094 s.e. to 403,690 ± 48,028 s.e. ($p = 0.008$).

In the thymus, E2-exposure did not significantly modulate the basal ROS/RNS-level of leucocytes (Fig. 5).

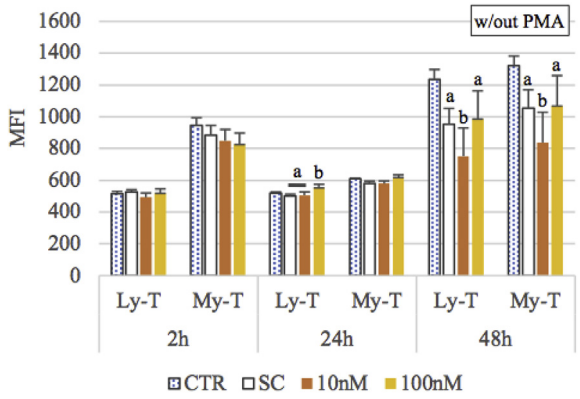

Fig. 5. ROS-level index (w/out, without PMA) of leucocytes isolated from the thymus (T), measured in lymphoid (Ly) and myeloid (My) cells after 2 h, 24 h and 48 h of primary cell culture without treatment (CTR), with 0.00005% ethanol (*i.e.*, solvent control, SC), with 10 nM and with 100 nM 17β-oestradiol. MFI, mean fluorescence index (for details see text). Values are means ± standard error of the means; different letters indicate statistically significant differences between SC, 10 nM and 100 nM groups at $p < 0.05$.

3.2.1.2. Twenty-four hours of E2-exposure. In the head-kidney, the E2-treatment decreased the proportion of oxidative burst positive myeloid cells at 10 nM from 47.2% ± 3.72 s.e. to 40.0% ± 2.89 s.e. ($p < 0.05$), but had no effect on the lymphoid cells (Fig. 4). The higher E2-concentration of 100 nM had no effect, neither on myeloid nor on lymphoid cells (Fig. 2). The ROS-level indices were significantly decreased by the E2-treatment at 10 and 100 nM (Fig. 4), both for the lymphoid cells (from 6.0 ± 0.5 s.e. to 4.1 ± 0.3 s.e., $p = 0.023$ and to 4.4 ± 0.6 s.e., $p = 0.029$; respectively) as well as for the myeloid cells (from 20.7 ± 2.8 s.e. to 8.2 ± 1.1 s.e. and to 8.1 ± 1.4 s.e., $p < 0.001$ for both concentrations). In contrast to 2 h of E2-exposure,

a significant increase of the basal ROS/RNS-level in both leucocyte populations was observed (lymphoid cells: from 4,240 ± 495 s.e. to 6,827 ± 794 s.e. (10 nM) and to 6,162 ± 535 s.e. (100 nM); myeloid cells: from 85,005 ± 13,191 s.e. to 150,281 ± 22,645 s.e. (10 nM) and to 155,601 ± 22,284 s.e. (100 nM), $p < 0.001$ for both cell types and both concentrations). The stimulated ROS-level, however, remained unchanged.

In the spleen, the E2-treatments had no significant effect on the proportion of oxidative burst positive myeloid cells (Fig. 2). The ROS-level index of lymphoid cells was significantly increased by E2 at 100 nM (from 3.27 ± 0.28 s.e. to 9.54 ± 2.25 s.e.; $p < 0.05$), but remained unchanged at 10 nM (Fig. 4). For the myeloid cells, however, E2 significantly decreased the ROS-level index at both concentrations (from 15.6 ± 2.5 s.e. to 4.5 ± 1.1 s.e., $p < 0.001$, at 10 nM, and to 8.0 ± 0.8 s.e., $p = 0.012$, at 100 nM). Furthermore, E2 significantly increased the basal ROS/RNS-level of myeloid cells at both concentrations (from 82,855 ± 12,454 s.e. to 145,616 ± 19,987 s.e., $p = 0.004$ for 10 nM and to 149,968 ± 21,355 s.e., $p = 0.006$ for 100 nM). Although no effect occurred at 10 nM, 100 nM E2 tended to increase the basal ROS-level of lymphoid cells (from 2,035 ± 290 s.e. to 2,814 ± 431 s.e., for 100 nM) without this difference being statistically significant. At 100 nM, E2-treatment tended to increase stimulated ROS-levels of lymphoid cells, from 6,358 ± 824 s.e. to 7,433 ± 1,068 s.e., whereas no effect occurred at 10 nM (6,925 ± 988 s.e.). In myeloid cells, 10 nM and 100 nM of E2 had no significant effect on the basal-ROS.

In the thymus, 100 nM E2 slightly, but significantly increased the basal ROS/RNS-level in the lymphocytes (from 499.9 ± 22.5 s.e. to 553.4 ± 21.6 s.e., $p < 0.001$), but had no effect at 10 nM (Fig. 5). No effect on myeloid cells was observed.

3.2.1.3. Forty-eight hours of E2-exposure. In the head-kidney, E2 significantly decreased the proportion of oxidative burst positive myeloid cells from 41.4% ± 3.62 s.e. to 28.7% ± 2.62 s.e. ($p < 0.05$) but had no effect at 100 nM (Fig. 2). Lymphoid cells remained unaffected by 10 and 100 nM E2 at this time point. Correspondingly, E2 significantly decreased the ROS-level indices of myeloid cells at both concentrations (from 7.4 ± 1.2 s.e. to 4.1 ± 0.6 s.e., $p < 0.001$ for 10 nM and to 3.2 ± 0.5 s.e., $p = 0.013$ for 100 nM), but did not affect the lymphoid cells (Fig. 4). The stimulated ROS-level of myeloid cells was significantly decreased by E2, although at 10 nM only (from 1,114,941 ± 125,334 s.e. to 747,460 ± 87,226 s.e., $p < 0.001$).

In the spleen, E2 had neither any significant effect on the respiratory burst capacity, nor on the ROS-level index of myeloid cells, or their basal ROS/RNS-levels at 48 h (Figs. 2 and 4). However, E2 at 10 nM significantly decreased the stimulated ROS-level of the myeloid cells (from 859,273 ± 105,905 s.e. to 578,953 ± 73,045 s.e., $p = 0.039$), but it had no effect at 100 nM.

In the thymus, 10 nM E2 significantly decreased the basal ROS/RNS-level in lymphoid cells from 953.1 ± 163.5 s.e. to 752.4 ± 99.5 s.e. and myeloid cells from 1,055.5 ± 188.3 s.e. to 837.8 ± 114.5 s.e. ($p < 0.05$ for both cell types) but had no significant effect at 100 nM (Fig. 5).

3.2.2. Leucocyte distribution and mortality

In both, the head-kidney and the spleen, E2-exposure at either concentration had no significant effect on leucocyte mortality and distribution (Figs. 6 and 7).

On the contrary, E2 significantly increased the mortality of lymphoid cells at 10 nM after 48 h in the thymus (from 2.07% ± 0.25 s.e. to 2.88% ± 0.26 s.e., $p = 0.006$). It had, however no effect on the myeloid cells (Fig. 6). The higher concentration of 100 nM E2 left the mortality of either cell type unaffected, but slightly decreased the proportion of lymphoid cells (Fig. 6) after 24 h and after 48 h (from 70.57% ± 2.31 s.e. to 69.01% ± 2.07 s.e., $p < 0.05$ and from 64.01% ± 2.45 s.e. to 63.19% ± 2.79 s.e., $p = 0.006$, respectively), resulting in an increased proportion of myeloid cells after 48 h of E2-exposure at 100 nM (from 27.4% ± 2.12 s.e. to 29.4% ± 0.02 s.e. $p = 0.009$).

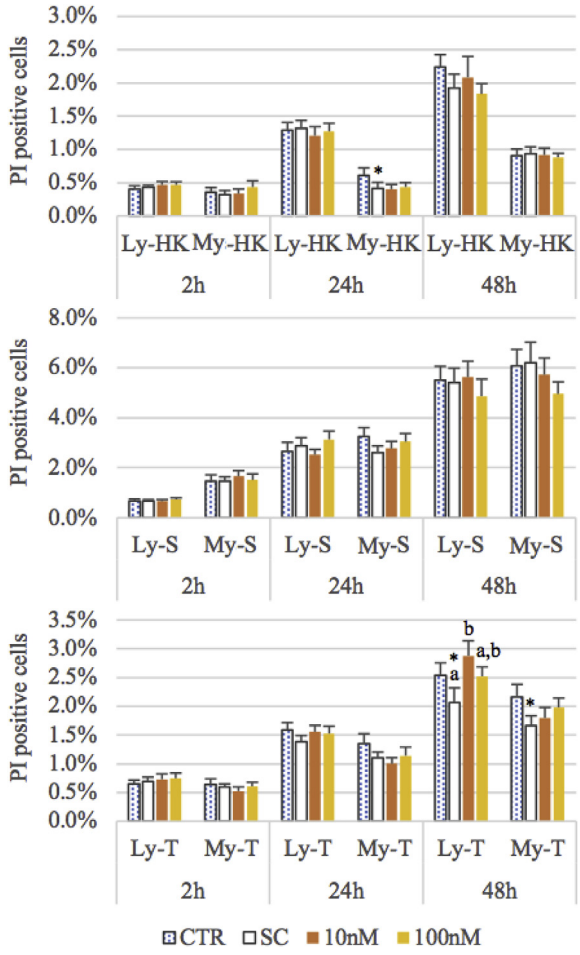

Fig. 6. Mortality of isolated leucocytes (*i.e.*, proportion of propidium iodide (PI) positive cells) from head-kidney (HK), spleen (S) and thymus (T) after 2 h, 24 h and 48 h of culture without treatment (CTR), with 0.0005‰ ethanol (*i.e.*, solvent control, SC), with 10 nM and 100 nM 17β-oestradiol. *, significantly different between the CTR and SC cells at $p < 0.05$; different letters indicate statistically significant differences between SC, 10 nM and 100 nM groups at $p < 0.05$.

4. Discussion

4.1. Signalling pathway of oestrogen in immune cells

4.1.1. Oestrogen receptor expression

Oestrogen receptors appear to be widely expressed at the transcript level in primary and secondary lymphoid organs of the European sea bass. All *Esrs* and *Gperb* were expressed in whole head-kidney and spleen as well as in the isolated leucocytes from these organs. This distribution is similar to the situation in another major lymphoid organ of sea bass, the thymus and the leucocytes isolated from the thymus, where the Esrs are strongly expressed [20]. Our results corroborate findings from other teleost species. Indeed, the expression of oestrogen receptors by various immune cells and immune organs has been reported for both, teleosts and mammals [40–43]. Importantly, our results indicate that E2 is able to directly modulate the immune cells of the head-kidney, the spleen and the thymus of sea bass by acting *via* genomic and non-genomic signalling pathways.

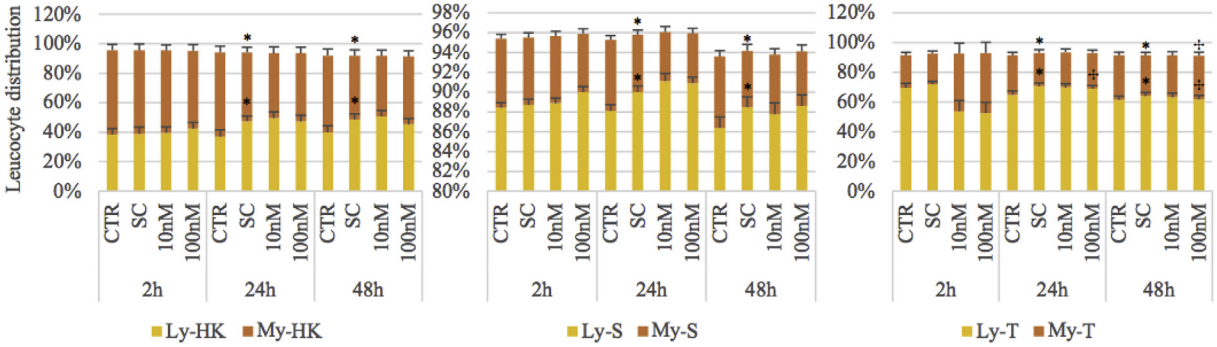

Fig. 7. Distribution of leucocytes derived from the head-kidney (HK), spleen (S) and thymus (T) after 2 h, 24 h and 48 h of culture without treatment (CTR), with 0.0005% ethanol (*i.e.*, solvent control, SC), with 10 nM and 100 nM 17β-oestradiol. The leucocyte distribution represents the percentage of events in the gates of lymphoid and myeloid cells. *, significantly different between the CTR and SC cells at $p < 0.05$. ✣, significantly different between SC and 100 nM groups at $p < 0.05$.

4.1.2. Non-genomic vs. genomic action

The rapid (2 h) changes in oxidative burst, basal ROS/RNS- and stimulated ROS-levels observed *in vitro* underscore the presumption of an E2-mediated regulation of immune cell activity in sea bass through membrane-associated oestrogen receptors in the head-kidney and the spleen. Such rapid E2-signalling is in line with findings in other teleosts and in mammals, where E2 has been described to modulate the respiratory burst of granulocytes and macrophages by acting on GPER [40,42,43]. In mammals, E2 also stimulates NO-production in human granulocytes and macrophages *via* non-genomic pathways [17]. As for lymphoid cells, oestrogen initiated a rapid cytosolic signalisation cascade, which resulted in the stimulation of T and B cell proliferation in mice [44,45]. Furthermore, as ROS- and Ca^{2+}-signalling are tightly linked with each other [46], it is possible that E2 also exerts a non-genomic regulation *via* changes in Ca^{2+}-fluxes, which further alter ROS-production. In addition, 24 h of E2-exposure increased the basal ROS/RNS-level in head-kidney leucocytes and in myeloid cells of the spleen. These late effects suggest that E2 modulates ROS/RNS-production and availability in lymphoid and myeloid cells of sea bass also *via* genomic signalling pathways. In fact, the modulation of ROS/RNS-levels in immune cells can result from an E2-mediated regulation of genes coding for anti-oxidant enzymes or for NO-synthase and NADPH-oxidase, as observed in mammals [17,47,48]. Accordingly, 6 h and 24 h *in vitro* exposure of red common carp macrophages to bisphenol A and S – two oestrogen agonists – modulates cytokine gene expression, *esr1*-expression as well as total ROS/RNS-level by modulating NO-synthase activity and total antioxidant capacity [49,50] by activating ESR-pathways [50].

4.2. Immunomodulatory action of oestrogen through specific regulation of redox biology

In the present work, E2-exposure resulted in opposed and transient effects on the basal ROS/RNS-level, in the different immune cell populations on the one hand, and at the different time points on the other hand. Indeed 2 h of E2-exposure transiently decreased the basal ROS/RNS-level of the splenic myeloid cells only and 24 h of E2-exposure transiently increased the ROS/RNS-levels in lymphoid cells of head-kidney and thymus as well as in myeloid cells of head-kidney and spleen. Our results are in line with those of Yang et al. (2015), who observed that 6 h and 24 h exposure to bisphenol A transiently increased total ROS/RNS-level of red common carp macrophages by activating ESR-pathways. In fact, these authors did neither observe any significant effects after 48 h of exposure, nor when using the ESR-antagonist ICI 182 780. In mammals, E2-mediated regulation of immune cells implies a modulation of the ROS/RNS-production and availability [17,47,48,51]. These ROS/RNS are involved in the differentiation, the apoptosis and the activity of various mammalian immune cells including lymphoid and myeloid cells [23–27]. Accordingly, our results suggest that in sea bass E2 directly and specifically modulates both lymphoid and myeloid cell activity/differentiation *via* a tight regulation of their redox biology comprising specific ROS/RNS-signalling as well as H_2O_2-permeability and NADPH-activation as described in the following sections.

4.2.1. Redox signalling in thymocyte and myeloid cell differentiation

In the thymus, 24 h of E2-exposure increased ROS/RNS-level in lymphoid cells, which is followed by an increase in mortality at 48 h as well as a decrease in the proportion of thymic lymphoid cells. Assuming that sea bass thymic leucocytes are mainly composed of T cells [52,53], this suggests that E2 stimulates immature T cell apoptosis by increasing ROS-levels. Indeed, oestradiol benzoate-injection has been reported to increase the number of degenerating lymphocytes in Masu salmon [54], which corroborates our interpretation. Similar observations have also been made in mammals, in which ROS are known to trigger the extrinsic or intrinsic pathway of T cell-apoptosis [23,24]. Furthermore, E2 was found to induce immature T cell apoptosis *in vivo* in mice [55,56]. Similarly, *in vitro* exposure of mice thymocytes to diethylstilbestrol – an oestrogen agonist – promoted programmed cell death [57]. Our interpretation that oestrogen modulates myeloid cell activity by promoting ROS/RNS-signalling also agrees with previous observations in mammals and teleosts, where E2 modulated the function of myeloid cells such as macrophages and granulocytes, both *in vitro* and *in vivo* [15,40,42,43,58]. In mammals, E2-signalling is also important for dendritic cell-differentiation and function [58,59]. Accordingly, Yang et al. (2013) and Qiu et al. (2018) observed that, in addition to modulate ROS/RNS-level, Bisphenol A- and S-exposure decreased antibacterial activity and cytokine expression of red common carp macrophages.

4.2.2. Lymphoid cell differentiation and H_2O_2-facilitated diffusion

Taking a closer look at the oxidative burst positive cells by employing appropriate cell gating revealed that a small fraction of lymphoid cells in the head-kidney (Fig. 2, Ly-HK) apparently displayed oxidative burst capacity. Indeed, mammalian and teleost B lymphocytes can display innate immune cell-like properties, such as antimicrobial, phagocytic and oxidative burst capacities [4,60,61]. No such population with oxidative burst capacity could be observed in the spleen (Fig. 2, Ly-S). Nonetheless, we detected that PMA increased the MFI of both lymphoid and myeloid cells of the spleen. This observation corresponds to earlier findings by Rabesandratana *et al.* (1992) [39]. Moreover, van Reyk *et al.* (2001) [62] observed that the intracellular increase of ROS-level in T cells arose from myeloid cells. In neutrophils, PMA stimulates both, the intracellular (inside granules) and the extracellular production of $O_2^{\bullet-}$ [37]. In other phagocytes as well as in B cells devoid of granules that are capable of generating intracellular $O_2^{\bullet-}$

, PMA is likely to stimulate extracellular $O_2^{\bullet-}$ only. The excreted $O_2^{\bullet-}$ is spontaneously converted into H_2O_2 [37]. Although H_2O_2 poorly diffuses across bilayer membranes, it can enter the cells by facilitated diffusion through aquaporins [63]. Interestingly, aquaporins and extracellular H_2O_2-uptake are both involved in phagocyte function as well as B and T lymphocyte activation, differentiation and migration [64–68]. It is, therefore, conceivable that the observed increase in ROS-levels of stimulated leucocytes result from extracellular H_2O_2 produced by the phagocytes, which enters the cells by facilitated diffusion. The fact that exogenous catalase inhibits the oxidative burst capacity may corroborate this interpretation [69]. Consequently, the significant increase of the ROS-level index observed after 24 h of 100 nM E2-exposure in lymphoid cells from the spleen would not result from a stimulation of the oxidative burst capacity, but rather derive from an E2-mediated increase of H_2O_2-diffusion through the plasmatic membrane. In fact, the basal ROS-level as well as the oxidative burst capacity of myeloid cells remained unaltered by E2 at this time point and concentration, thus substantiating this explanation. Consequently, the E2-mediated increase of the ROS-level index in the lymphoid cells of the spleen at 24 h suggests an E2 stimulated aquaporin-expression by inducing lymphocyte activation and differentiation [64,66]. That E2 indeed promotes lymphocyte differentiation is suggested by the time point (24 h) at which the stimulation occurs. Moreover, observations in fish and mammalian T and B lymphocytes after oestrogen exposure [17,19,70] corroborate this interpretation. Indeed, Rodenas et al. (2017a), for instance, found a boost in B lymphocyte-differentiation and activity (antibody production) following *in vivo*-exposure to ethinylestradiol and G-1, a GPER-specific agonist. Moreover, E2 also enhances peripheral B cell differentiation and activity in the mammalian spleen [17,58,71].

4.2.3. Oxidative burst capacity and NADPH-oxidase activation

Exposure to E2 at 10 nM for 2 h significantly inhibited the oxidative burst capacity and resulted in a decrease of the stimulated ROS-level. The former was only observed in head-kidney leucocytes, the latter also occurred in the myeloid cells of the spleen at 2 h of exposure, but to a far lesser extent and only at the highest E2-levels. These observations suggest that, in sea bass, E2 rapidly inhibited PMA-mediated activation of ROS-production, *i.e.*, NADPH-oxidase activation. This effect on the oxidative burst capacity was still significant after 24 h and 48 h of treatment, but only at 10 nM and only in the myeloid cells of the head-kidney. In agreement Cabas et *al.* 2012 and Liarte et *al.* 2011 observed that *in vitro* E2-exposure inhibited VaDNA-stimulated oxidative burst [72,73]. Although this appears to contradict the results of numerous other studies, which describe a stimulation of the respiratory burst by E2 [32,40,43,74], the results are not readily comparable. The numerous studies differ in their experimental design and in the culture conditions for the most part. Cells were obtained from different species at different seasons and various degrees of sexual maturation. Most importantly, however, the assessment of ROS-levels differed across the studies. Notably the ROS-sensitive probes measuring intracellular and/or extracellular ROS are quite diverse [75]. The same holds true for the time-kinetics [40]. Furthermore, the normalisation method can lead to very different conclusions. In fact, the measure of oxidative burst capacity based on the ROS-level index may be biased if E2 affects the basal RNS/ROS-level: in the case of an E2-mediated increase of the basal RNS/ROS-level, the overall respiratory burst index decreases, whereas a decrease of the basal RNS/ROS-level would result in an over-estimation of the respiratory burst index. This may be exemplified by the results for the splenocytes after 24 h of exposure, which result in a decrease of the ROS-level indices without a noticeable effect on the proportions of oxidative burst positive cells. Such changes in the basal values are, however, not reported very often, even if the intracellular level of ROS is measured. This makes it difficult to evaluate the parameters that affect the respiratory burst index, in the first place. To validate the method employed in the present work, we reassessed published data obtained after exposure of sea bass to E2 *in vivo* [19]. The experience from this earlier study showed that intraperitoneal E2-injections over one week significantly decreased the proportion of both oxidative burst positive myeloid and lymphoid cells in the spleen. When using ROS-level indices based on the ratio of stimulated and basal RNS/ROS-levels, however, E2-treatment had a significant inhibitory effect only on the lymphoid cells, but not myeloid cells. Hence, this method allows for a more accurate and differentiated analysis.

5. Conclusion

The presence of oestrogen receptors in immune cells as well as the results from the *in vitro* E2-exposure of leucocytes isolated from the head-kidney, the spleen and the thymus clearly indicated that E2 directly and specifically modulates ROS/RNS-production and signalling in leucocytes from primary and secondary lymphoid organs. The presence of both, nuclear and membrane oestrogen receptors, points to E2-signalling *via* genomic and non-genomic pathways. These two major signalling pathways are reflected by short-term (2 h) and later (24 h–48 h) ROS/RNS-responses of the leucocytes. Both types of receptors, present as multiple isoforms in the investigated immune cells, are likely responsible for complex and context-dependent immunomodulatory functions of E2, depending on the E2-concentration, the time kinetics, the lymphoid organs, the leucocyte population and their developmental stage. Hence this study confirms variability in oestrogenic immune cell responsiveness for fish. Importantly, our study suggests that the situation- and concentration-dependent immunomodulatory action of oestrogen is mediated by a tight and direct regulation of the redox status in immune cells including ROS/RNS-signalling, NADPH-oxidase activity and H_2O_2-permeability.

Additionally, our results indicate that, in sea bass, the head-kidney and spleen leucocytes have a different E2-sensitive oxidative burst capacity, which is likely to be due to a different composition in immune cell types. It is, therefore, assumed that E2 does not directly promote inhibition of the oxidative burst capacity in the spleen of sea bass, but rather indirectly by promoting regulatory T cell differentiation *in vivo* [19].

In summary, this study confirms the complex immunomodulatory function of E2 in teleosts, involving (1) different mechanisms of action with distinct kinetics and endpoints, (2) a tight regulation of the leucocyte redox biology as well as (3) a specific modulation of immune cells from both primary and secondary lymphoid organs. Oestrogens, therefore, regulate immune cell differentiation (*i.e.*, immature T cell fate) and function as they do in mammals. Overall, the findings strengthen the idea of a conserved role of oestrogens in immunomodulation in all vertebrates.

Compliance with ethical standards

All applicable national guidelines for the care and use of animals were followed.

Conflicts of interest

The authors declare that they have no conflict of interest.

Acknowledgements

This research was supported by the ANR financed project ETaT (ANR-15-CE32-0014). M.P. was supported by the Normandy region through the FR CNRS 3730 SCALE. A PhD research fellowship allocated by the French Ministry of Higher Education and Research to Le Havre University funded C.M. The authors thank Matthieu Bonnet and the staff at Aquacaux for fish hatchery.

References

[1] T. Boehm, J.B. Swann, Origin and evolution of adaptive immunity, Annu. Rev. Anim. Biosci. 2 (2014) 259–283, https://doi.org/10.1146/annurev-animal-022513-114201.

[2] L. Pasman, D.L. Kasper, Building conventions for unconventional lymphocytes, Immunol. Rev. 279 (2017) 52–62, https://doi.org/10.1111/imr.12576.

[3] M.Á. Esteban, A. Cuesta, E. Chaves-Pozo, J. Meseguer, Phagocytosis in teleosts. Implications of the new cells involved, Biology 4 (2015) 907–922, https://doi.org/10.3390/biology4040907.

[4] M. Riera Romo, D. Pérez-Martínez, C. Castillo Ferrer, Innate immunity in vertebrates: an overview, Immunology 148 (2016) 125–139, https://doi.org/10.1111/imm.12597.

[5] D. Carbone, C. Faggio, Importance of prebiotics in aquaculture as immunostimulants. Effects on immune system of *Sparus aurata* and *Dicentrarchus labrax*, Fish Shellfish Immunol. 54 (2016) 172–178, https://doi.org/10.1016/j.fsi.2016.04.011.

[6] E.R. Lauriano, C. Faggio, G. Capillo, N. Spanò, M. Kuciel, M. Aragona, S. Pergolizzi, Immunohistochemical characterization of epidermal dendritic-like cells in giant mudskipper, *Periophthalmodon schlosseri*, Fish Shellfish Immunol. 74 (2018) 380–385, https://doi.org/10.1016/j.fsi.2018.01.014.

[7] D. Parra, F. Takizawa, J.O. Sunyer, Evolution of B cell immunity, Annu. Rev. Anim. Biosci. 1 (2013) 65–97, https://doi.org/10.1146/annurev-animal-031412-103651.

[8] C. Tafalla, E. Leal, T. Yamaguchi, U. Fischer, T cell immunity in the teleost digestive tract, Dev. Comp. Immunol. 64 (2016) 167–177, https://doi.org/10.1016/j.dci.2016.02.019.

[9] J. Havixbeck, D. Barreda, Neutrophil development, migration, and function in teleost fish, Biology 4 (2015) 715–734, https://doi.org/10.3390/biology4040715.

[10] A. Sfacteria, M. Brines, U. Blank, The mast cell plays a central role in the immune system of teleost fish, Mol. Immunol. 63 (2015) 3–8, https://doi.org/10.1016/j.molimm.2014.02.007.

[11] G.W. Litman, J.P. Rast, S.D. Fugmann, The origins of vertebrate adaptive immunity, Nat. Rev. Immunol. 10 (2010) 543–553, https://doi.org/10.1038/nri2807.

[12] M.A. Burgos-Aceves, A. Cohen, Y. Smith, C. Faggio, Estrogen regulation of gene expression in the teleost fish immune system, Fish Shellfish Immunol. 58 (2016) 42–49, https://doi.org/10.1016/j.fsi.2016.09.006.

[13] H. Segner, B.M.L. Verburg-van Kemenade, M. Chadzinska, The immunomodulatory role of the hypothalamus-pituitary-gonad axis: proximate mechanism for reproduction-immune trade offs? Dev. Comp. Immunol. 66 (2017) 43–60, https://doi.org/10.1016/j.dci.2016.07.004.

[14] H. Segner, A. Casanova-Nakayama, R. Kase, C.R. Tyler, Impact of environmental estrogens on fish considering the diversity of estrogen signaling, Gen. Comp. Endocrinol. 191 (2013) 190–201, https://doi.org/10.1016/j.ygcen.2013.05.015.

[15] E. Szwejser, B.M.L. Verburg-van Kemenade, M. Maciuszek, M. Chadzinska, Estrogen-dependent seasonal adaptations in the immune response of fish, Horm. Behav. (2016), https://doi.org/10.1016/j.yhbeh.2016.10.007.

[16] S. Oertelt-Prigione, Immunology and the menstrual cycle, Autoimmun. Rev. 11 (2012) A486–A492, https://doi.org/10.1016/j.autrev.2011.11.023.

[17] R.H. Straub, The complex role of estrogens in inflammation, Endocr. Rev. 28 (2007) 521–574, https://doi.org/10.1210/er.2007-0001.

[18] C.R. Wira, M. Rodriguez-Garcia, M.V. Patel, The role of sex hormones in immune protection of the female reproductive tract, Nat. Rev. Immunol. 15 (2015) 217–230, https://doi.org/10.1038/nri3819.

[19] M. Paiola, T. Knigge, A. Duflot, P.I.S. Pinto, E. Farcy, T. Monsinjon, Oestrogen, an evolutionary conserved regulator of T cell differentiation and immune tolerance in jawed vertebrates? Dev. Comp. Immunol. 84 (2018) 48–61, https://doi.org/10.1016/j.dci.2018.01.013.

[20] M. Paiola, T. Knigge, S. Picchietti, A. Duflot, L. Guerra, P.I.S. Pinto, G. Scapigliati, T. Monsinjon, Oestrogen receptor distribution related to functional thymus anatomy of the European sea bass, Dicentrarchus labrax, Dev. Comp. Immunol. (2017), https://doi.org/10.1016/j.dci.2017.07.023.

[21] E. Chaves-Pozo, A. García-Ayala, I. Cabas, Effects of sex steroids on fish leukocytes, Biology 7 (2018) 9, https://doi.org/10.3390/biology7010009.

[22] D. Khan, S. Ansar Ahmed, The immune system is a natural target for estrogen action: opposing effects of estrogen in two prototypical autoimmune diseases, Front. Immunol. 6 (2016), https://doi.org/10.3389/fimmu.2015.00635.

[23] J.M. Gostner, K. Becker, D. Fuchs, R. Sucher, Redox regulation of the immune response, Redox Rep. 18 (2013) 88–94, https://doi.org/10.1179/1351000213Y.0000000044.

[24] Y. Yang, A.V. Bazhin, J. Werner, S. Karakhanova, Reactive oxygen species in the immune system, Int. Rev. Immunol. 32 (2013) 249–270, https://doi.org/10.3109/08830185.2012.755176.

[25] A. García-Ortiz, J.M. Serrador, Nitric oxide signaling in T cell-mediated immunity, Trends Mol. Med. 24 (2018) 412–427, https://doi.org/10.1016/j.molmed.2018.02.002.

[26] C. Nathan, A. Cunningham-Bussel, Beyond oxidative stress: an immunologist's guide to reactive oxygen species, Nat. Rev. Immunol. 13 (2013) 349–361, https://doi.org/10.1038/nri3423.

[27] P. Thwe, E. Amiel, The role of nitric oxide in metabolic regulation of dendritic cell immune function, Cancer Lett. (2017), https://doi.org/10.1016/j.canlet.2017.10.032.

[28] M.A. Burgos-Aceves, A. Cohen, Y. Smith, C. Faggio, MicroRNAs and their role on fish oxidative stress during xenobiotic environmental exposures, Ecotoxicol. Environ. Saf. 148 (2018) 995–1000, https://doi.org/10.1016/j.ecoenv.2017.12.001.

[29] M. Timm, L. Saaby, L. Moesby, E.W. Hansen, Considerations regarding use of solvents in in vitro cell based assays, Cytotechnology 65 (2013) 887–894, https://doi.org/10.1007/s10616-012-9530-6.

[30] A.G. Granja, E. Leal, J. Pignatelli, R. Castro, B. Abos, G. Kato, U. Fischer, C. Tafalla, Identification of teleost skin CD8 + dendritic-like cells, representing a potential common ancestor for mammalian cross-presenting dendritic cells, J. Immunol. 195 (2015) 1825–1837, https://doi.org/10.4049/jimmunol.1500322.

[31] F. Seemann, T. Knigge, A. Duflot, S. Marie, S. Olivier, C. Minier, T. Monsinjon, Sensitive periods for 17β-estradiol exposure during immune system development in sea bass head kidney: sensitivity of sea bass head kidney development to E2, J. Appl. Toxicol. 36 (2016) 815–826, https://doi.org/10.1002/jat.3215.

[32] A. Bado-Nilles, R. Techer, J.M. Porcher, A. Geffard, B. Gagnaire, S. Betoulle, W. Sanchez, Detection of immunotoxic effects of estrogenic and androgenic endocrine disrupting compounds using splenic immune cells of the female three-spined stickleback, *Gasterosteus aculeatus* (L.), Environ. Toxicol. Pharmacol. 38 (2014) 672–683, https://doi.org/10.1016/j.etap.2014.08.002.

[33] G.T. Haugland, A. Rønneseth, H.I. Wergeland, Flow cytometry analyses of phagocytic and respiratory burst activities and cytochemical characterization of leucocytes isolated from wrasse (*Labrus bergylta* A.), Fish Shellfish Immunol. 39 (2014) 51–60, https://doi.org/10.1016/j.fsi.2014.04.023.

[34] X. Chen, Z. Zhong, Z. Xu, L. Chen, Y. Wang, 2′,7′-Dichlorodihydrofluorescein as a fluorescent probe for reactive oxygen species measurement: forty years of application and controversy, Free Radic. Res. 44 (2010) 587–604, https://doi.org/10.3109/10715761003709802.

[35] S. Dupre-Crochet, M. Erard, O. Nusse, ROS production in phagocytes: why, when, and where? J. Leukoc. Biol. 94 (2013) 657–670, https://doi.org/10.1189/jlb.1012544.

[36] I. Peluso, H. Manafikhi, R. Reggi, M. Palmery, Interference of flavonoids with fluorescent intracellular probes: methodological implications in the evaluation of the oxidative burst by flow cytometry, Cytometry 85 (2014) 663–677, https://doi.org/10.1002/cyto.a.22490.

[37] J. Bylund, K.L. Brown, C. Movitz, C. Dahlgren, A. Karlsson, Intracellular generation of superoxide by the phagocyte NADPH oxidase: how, where, and what for? Free Radic. Biol. Med. 49 (2010) 1834–1845, https://doi.org/10.1016/j.freeradbiomed.2010.09.016.

[38] I. Peluso, G. Morabito, S. Riondino, F. La Farina, M. Serafini, Lymphocytes as internal standard in oxidative burst analysis by cytometry: a new data analysis approach, J. Immunol. Methods 379 (2012) 61–65, https://doi.org/10.1016/j.jim.2012.02.015.

[39] H. Rabesandratana, A.-M. Fournier, M.-T. Chateau, A. Serre, J. Dornand, Increased oxidative metabolism in PMA-activated lymphocytes: a flow cytometric study, Int. J. Immunopharm. 14 (1992) 895–902 https://doi.org/10.1016/0192-0561(92)90089-4.

[40] I. Cabas, M.C. Rodenas, E. Abellan, J. Meseguer, V. Mulero, A. Garcia-Ayala, Estrogen signaling through the G protein-coupled estrogen receptor regulates granulocyte activation in fish, J. Immunol. 191 (2013) 4628–4639, https://doi.org/10.4049/jimmunol.1301613.

[41] A.-G. Lafont, K. Rousseau, J. Tomkiewicz, S. Dufour, Three nuclear and two membrane estrogen receptors in basal teleosts, Anguilla sp.: identification, evolutionary history and differential expression regulation, Gen. Comp. Endocrinol. 235 (2016) 177–191, https://doi.org/10.1016/j.ygcen.2015.11.021.

[42] M.C. Rodenas, N. Tamassia, I. Cabas, F. Calzetti, J. Meseguer, M.A. Cassatella, A. García-Ayala, V. Mulero, G protein-coupled estrogen receptor 1 regulates human neutrophil functions, Biomed. Hub. 2 (2017), https://doi.org/10.1159/000454981 2–2.

[43] E. Szwejser, M. Maciuszek, A. Casanova-Nakayama, H. Segner, B.M.L. Verburg-van Kemenade, M. Chadzinska, A role for multiple estrogen receptors in immune regulation of common carp, Dev. Comp. Immunol. 66 (2017) 61–72, https://doi.org/10.1016/j.dci.2016.04.003.

[44] M. Ádori, E. Kiss, Z. Barad, K. Barabás, E. Kiszely, A. Schneider, E. Sziksz, I.M. Ábrahám, J. Matkó, G. Sármay, Estrogen augments the T cell-dependent but not the T-independent immune response, Cell. Mol. Life Sci. 67 (2010) 1661–1674, https://doi.org/10.1007/s00018-010-0270-5.

[45] A.E. Schneider, É. Kárpáti, K. Schuszter, E.A. Tóth, E. Kiss, M. Kulcsár, G. László, J. Matko, A dynamic network of estrogen receptors in murine lymphocytes: fine-tuning the immune response, J. Leukoc. Biol. 96 (2014) 857–872, https://doi.org/10.1189/jlb.2A0214-080RR.

[46] A. Görlach, K. Bertram, S. Hudecova, O. Krizanova, Calcium and ROS: a mutual interplay, Redox Biol. 6 (2015) 260–271, https://doi.org/10.1016/j.redox.2015.08.010.

[47] E. Karpuzoglu, S.A. Ahmed, Estrogen regulation of nitric oxide and inducible nitric oxide synthase (iNOS) in immune cells: implications for immunity, autoimmune diseases, and apoptosis, Nitric Oxide 15 (2006) 177–186, https://doi.org/10.1016/j.niox.2006.03.009.

[48] H.P. Priyanka, H.C. Krishnan, R.V. Singh, L. Hima, S. ThyagaRajan, Estrogen modulates in vitro T cell responses in a concentration- and receptor-dependent manner: effects on intracellular molecular targets and antioxidant enzymes, Mol. Immunol. 56 (2013) 328–339, https://doi.org/10.1016/j.molimm.2013.05.226.

[49] W. Qiu, M. Yang, S. Liu, P. Lei, L. Hu, B. Chen, M. Wu, K.-J. Wang, Toxic effects of bisphenol S showing immunomodulation in fish macrophages, Environ. Sci. Technol. 52 (2018) 831–838, https://doi.org/10.1021/acs.est.7b04226.

[50] M. Yang, W. Qiu, B. Chen, J. Chen, S. Liu, M. Wu, K.-J. Wang, The in vitro immune modulatory effect of bisphenol a on fish macrophages via estrogen receptor α and nuclear factor-κB signaling, Environ. Sci. Technol. 49 (2015) 1888–1895, https://doi.org/10.1021/es505163v.

[51] T. Pan, L. Zhong, S. Wu, Y. Cao, Q. Yang, Z. Cai, X. Cai, W. Zhao, N. Ma, W. Zhang,

H. Zhang, J. Zhou, 17β-Oestradiol enhances the expansion and activation of myeloid-derived suppressor cells via signal transducer and activator of transcription (STAT) − 3 signalling in human pregnancy, Clin. Exp. Immunol. 185 (2016) 86–97, https://doi.org/10.1111/cei.12790.
[52] N. Romano, L. Abelli, L. Mastrolia, G. Scapigliati, Immunocytochemical detection and cytomorphology of lymphocyte subpopulations in a teleost fish Dicentrarchus labrax, Cell Tissue Res. 289 (1997) 163–171 https://doi.org/10.1007/s004410050862.
[53] G. Scapigliati, M. Mazzini, L. Mastrolia, N. Romano, L. Abelli, Production and characterisation of a monoclonal antibody against the thymocytes of the sea bass Dicentrarchus labrax (L.) (Teleostea, Percicthydae), Fish Shellfish Immunol. 5 (1995) 393–405, https://doi.org/10.1006/fsim.1995.0039.
[54] B.G. Sufi, K. Mori, I. Nomura, Involution of thymus in relation to sexual and steroid hormone treatments in salmonid fish, Tohoku J. Agric. Res. 31 (1980) 97–105.
[55] Y. Do, S. Ryu, M. Nagarkatti, P.S. Nagarkatti, Role of death receptor pathway in estradiol-induced T-cell apoptosis in vivo, Toxicol. Sci. 70 (2002) 63–72, https://doi.org/10.1093/toxsci/70.1.63.
[56] A. Martín, L.M. Alonso, M.G. Del Moral, A.G. Zapata, Ultrastructural changes in the adult rat thymus after estradiol benzoate treatment, Tissue Cell 26 (1994) 169–179, https://doi.org/10.1016/0040-8166(94)90092-2.
[57] L. Leung-Gurung, P. Escalante Cobb, F. Mourad, C. Zambrano, Z. Muscato, V. Sanchez, K. Godde, C. Broussard, Methoxychlor metabolite HPTE alters viability and differentiation of embryonic thymocytes from C57BL/6 mice, J. Immunot. 15 (2018) 104–118, https://doi.org/10.1080/1547691X.2018.1474978.
[58] J. Roved, H. Westerdahl, D. Hasselquist, Sex differences in immune responses: hormonal effects, antagonistic selection, and evolutionary consequences, Horm. Behav. 88 (2017) 95–105, https://doi.org/10.1016/j.yhbeh.2016.11.017.
[59] S. Laffont, C. Seillet, J.-C. Guéry, Estrogen receptor-dependent regulation of dendritic cell development and function, Front. Immunol. 8 (2017), https://doi.org/10.3389/fimmu.2017.00108.
[60] I. Kovacs, M. Horvath, A. Lanyi, G.L. Petheo, M. Geiszt, Reactive oxygen species-mediated bacterial killing by B lymphocytes, J. Leukoc. Biol. 97 (2015) 1133–1137, https://doi.org/10.1189/jlb.4AB1113-607RR.
[61] X.-J. Zhang, P. Wang, N. Zhang, D.-D. Chen, P. Nie, J.-L. Li, Y.-A. Zhang, B cell functions can Be modulated by antimicrobial peptides in rainbow trout Oncorhynchus mykiss: novel insights into the innate nature of B cells in fish, Front. Immunol. 8 (2017), https://doi.org/10.3389/fimmu.2017.00388.
[62] D.M. van Reyk, N.J.C. King, M.C. Dinauer, N.H. Hunt, The intracellular oxidation of 2′,7′-dichlorofluorescin in murine T lymphocytes, Free Radic. Biol. Med. 30 (2001) 82–88, https://doi.org/10.1016/S0891-5849(00)00449-4.
[63] G.P. Bienert, F. Chaumont, Aquaporin-facilitated transmembrane diffusion of hydrogen peroxide, Biochim. Biophys. Acta BBA - Gen. Subj. 1840 (2014) 1596–1604, https://doi.org/10.1016/j.bbagen.2013.09.017.
[64] M. Bertolotti, G. Farinelli, M. Galli, A. Aiuti, R. Sitia, AQP8 transports NOX2-generated H_2O_2 across the plasma membrane to promote signaling in B cells, J. Leukoc. Biol. 100 (2016) 1071–1079, https://doi.org/10.1189/jlb.2AB0116-045R.
[65] M. Hara-Chikuma, S. Chikuma, Y. Sugiyama, K. Kabashima, A.S. Verkman, S. Inoue, Y. Miyachi, Chemokine-dependent T cell migration requires aquaporin-3–mediated hydrogen peroxide uptake, J. Exp. Med. 209 (2012) 1743–1752, https://doi.org/10.1084/jem.20112398.
[66] C. Moon, R. Rousseau, J.-C. Soria, M.O. Hoque, J. Lee, S.J. Jang, B. Trink, D. Sidransky, L. Mao, Aquaporin expression in human lymphocytes and dendritic cells, Am. J. Hematol. 75 (2004) 128–133, https://doi.org/10.1002/ajh.10476.
[67] D. Tyteca, T. Nishino, H. Debaix, P.V.D. Smissen, F. N'Kuli, D. Hoffmann, Y. Cnops, V. Rabolli, G. van Loo, R. Beyaert, F. Huaux, O. Devuyst, P.J. Courtoy, Regulation of macrophage motility by the water channel aquaporin-1: crucial role of M0/M2 phenotype switch, PloS One 10 (2015), https://doi.org/10.1371/journal.pone.0117398 e0117398.
[68] N. Zhu, X. Feng, C. He, H. Gao, L. Yang, Q. Ma, L. Guo, Y. Qiao, H. Yang, T. Ma, Defective macrophage function in aquaporin-3 deficiency, Faseb. J. 25 (2011) 4233–4239, https://doi.org/10.1096/fj.11-182808.
[69] J. Ortuño, M. Esteban, J. Meseguer, Kinetics of hydrogen peroxide production during in vitro respiratory burst of seabream (*Sparus aurata* L.) head-kidney leucocytes, as measured by a flow cytometric method, Fish Shellfish Immunol. 10 (2000) 725–729, https://doi.org/10.1006/fsim.2000.0285.
[70] M.C. Rodenas, I. Cabas, N.E. Gómez-González, M. Arizcun, J. Meseguer, V. Mulero, A. García-Ayala, Estrogens promote the production of natural neutralizing antibodies in fish through G protein-coupled estrogen receptor 1, Front. Immunol. 8 (2017), https://doi.org/10.3389/fimmu.2017.00736.
[71] A.I. Bernardi, A. Andersson, L. Grahnemo, M. Nurkkala-Karlsson, C. Ohlsson, H. Carlsten, U. Islander, Effects of lasofoxifene and bazedoxifene on B cell development and function, Immun. Inflamm. Dis. 2 (2014) 214–225, https://doi.org/10.1002/iid3.37.
[72] S. Liarte, E. Chaves-Pozo, E. Abellán, J. Meseguer, V. Mulero, A. García-Ayala, 17β-Estradiol regulates gilthead seabream professional phagocyte responses through macrophage activation, Dev. Comp. Immunol. 35 (2011) 19–27, https://doi.org/10.1016/j.dci.2010.07.007.
[73] I. Cabas, S. Liarte, A. García-Alcázar, J. Meseguer, V. Mulero, A. García-Ayala, 17α-Ethynylestradiol alters the immune response of the teleost gilthead seabream (*Sparus aurata* L.) both in vivo and in vitro, Dev. Comp. Immunol. 36 (2012) 547–556, https://doi.org/10.1016/j.dci.2011.09.011.
[74] L.R. Iwanowicz, J.L. Stafford, R. Patiño, E. Bengten, N.W. Miller, V.S. Blazer, Channel catfish (*Ictalurus punctatus*) leukocytes express estrogen receptor isoforms ERα and ERβ2 and are functionally modulated by estrogens, Fish Shellfish Immunol. 40 (2014) 109–119, https://doi.org/10.1016/j.fsi.2014.06.021.
[75] J. Bylund, H. Björnsdottir, M. Sundqvist, A. Karlsson, C. Dahlgren, Measurement of respiratory burst products, released or retained, during activation of professional phagocytes, Neutrophil Methods Protoc, Humana Press, Totowa, NJ, 2014, pp. 321–338, , https://doi.org/10.1007/978-1-62703-845-4_21.